Targeted Expression of c-Src in Epidermal Basal Cells Leads to Enhanced Skin Tumor Promotion, Malignant Progression, and Metastasis

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ABSTRACT

In this study, we generated transgenic mice that overexpressed either a constitutively active human c-src mutant (src530) or a wild-type human c-src (srcwt) in epidermal basal cells driven by human keratin 14 (HK14) or bovine keratin 5 (BK5) promoters, respectively. HK14.src530 transgenic mice developed severe epidermal hyperplasia and hyperkeratosis, and did not survive beyond 3 weeks of age. Four transgenic founders were obtained after injection of a BK5.srcwt construct with variable phenotypes, and three lines (lines A-C) were established. BK5.srcwt founder D exhibited a severe skin phenotype similar to HK14.src530 transgenic mice and died 5 days after birth. Line C transgenic mice also exhibited significant epidermal hyperplasia and hyperkeratosis, and developed spontaneous squamous cell carcinomas (SCCs) of the skin beginning at ~3 months of age (70% incidence at 1 year). Mice from lines A and B did not show a marked phenotype; however, elevated human src protein in the epidermis of line B mice was clearly evident. Additional analyses of line B transgenic mice showed an enhanced responsiveness to 12-O-tetradecanoylphorbol-13-acetate-induced epidermal hyperplasia and cell proliferation. Analysis of the susceptibility of line B mice to two-stage skin carcinogenesis revealed that papillomas and SCCs arose earlier and in greater numbers compared with nontransgenic littermates. In addition, malignant conversion occurred more rapidly, and the SCCs that developed in line B transgenic mice had a greater propensity to metastasize to peripheral lymph nodes and other organs. These observations support the hypothesis that c-src plays an important role in skin tumor promotion. In addition, the data show that elevated c-src activity enhances malignant progression and metastasis in this model system.

INTRODUCTION

The c-src gene is the cellular homologue of v-src, which was first identified in the genome of the oncogenic retrovirus, Rous sarcoma virus (1). The c-src gene encodes an intracellular tyrosine kinase with a molecular weight of M, 60,000 (2). c-src contains src homology domains 2 and 3, a catalytic region, and a negative-regulatory tyrosine residue (tyr 527 in chicken, tyr 529 in mouse, and tyr 530 in human) located near the COOH terminus (3). c-src is the prototype member of a family of intracellular nonreceptor tyrosine kinases (4). Other family members that share similar structural and biochemical properties include fyn, yes, lyn, hck, lyn, and yrk (5). Three of these family members, src, fyn, and yes, are widely expressed in tissues and appear to play an important role in regulation of cell adhesion, cell growth, and differentiation (6). The src tyrosine kinases have long been established as potential oncopogens. v-src and mutated forms of c-src are capable of transforming many different cell types (7). Activation or overexpression of human c-src has been observed in human tumors including breast, colon, bladder, stomach, ovary, lung, prostate, and pancreas (8–11).

In our previous work, src kinase activity was found to be elevated in cultured mouse keratinocytes exposed to EGF (3) and in the epidermis of SENCAR mice treated with TPA (12). Activation of c-src kinase also was observed in the epidermis of TGF-α transgenic mice where expression of human TGF-α was targeted to basal keratinocytes with the HK14 promoter (12). In addition, we recently generated HK14.src529 transgenic mice that overexpress a constitutively active mouse c-src mutant (src529) in interfollicular epidermis driven by the HK1 promoter (13). The src529 mutant has the major negative-regulatory tyrosine residue at position 529 replaced with phenylalanine; thus, it cannot be regulated by COOH-terminal src kinase (3, 5). These mice had epidermal hyperplasia and hyperkeratosis for ~1 week after birth and displayed enhanced susceptibility to tumor promotion by TPA. These data suggested that activation of c-src plays a role in the promotion stage of multistage skin tumorigenesis. However, the skin phenotype subsided with the cessation of K1 expression, and no spontaneous skin tumors arose in adult HK1.src529 transgenic mice.

In this study, we generated c-src transgenic mice with targeted expression in the basal layer of the epidermis to obtain transgenic mice with a more persistent phenotype. HK14.src530 transgenic mice, which expressed a constitutively active human c-src mutant (src530) in epidermal basal cells, developed severe epidermal hyperplasia and did not survive beyond 3 weeks of age. Subsequently, transgenic mice were generated that overexpressed a wild-type human c-src (srcwt) in the epidermal basal cells driven by the BK5 promoter (BK5.srcwt mice). From this construct, four transgenic founders were obtained with various phenotypes. In this report, we describe the skin phenotype of these mice and the susceptibility of one line (BK5.srcwt line B) to two-stage carcinogenesis. The results confirm that c-src plays an important role in the process of skin tumor promotion. In addition, malignant conversion occurred more rapidly in BK5.srcwt line B mice, and the SCCs that developed in these mice exhibited a higher propensity for metastasis to peripheral lymph nodes and other organs. These observations support the hypothesis that c-src also plays an important role in malignant progression and metastasis in this model system.

MATERIALS AND METHODS

Preparation of DNA Constructs. Plasmid DNA manipulations were performed by procedures described previously (14). The DNA construct used for generating HK14.src530 transgenic mice is shown in Fig. I. A human c-src Y530F cDNA was excised from the parent pMT-CB6 530F plasmid using HinfII and BamHI restriction endonucleases. The termini of this fragment

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were filled in with the Klenow fragment of DNA polymerase, and the blunt-ended fragment was ligated into the SnaBI site of a pK14 keratinocyte expression vector between a generic intron (which consisted of a 5’ untranslated region of an adenovirus major late gene containing an mRNA cap site and splice donor) and a 3’ region of mouse IgG containing a splicing donor and a 3’/H11032 polyadenylation signal. The DNA construct used for generating BK5.srcwt transgenic mice is shown in Fig. 2A. A human c-src cDNA was excised from the parent MT-CB6 c-src plasmid using HindIII and BamH1 restriction endonucleases, and inserted into the BK5 keratinocyte expression vector described previously (14). Orientation and integrity of both cDNA inserts were determined by a series of diagnostic restriction digests.

Preparation of DNA for Microinjection. The HK14.src530 and BK5.srcwt vectors were digested with SaeI to release the vector sequences from the expression construct. The resulting fragment was separated by electrophoresis through an 0.8% agarose gel. The fragment containing the expression construct was isolated from a gel slice by a PCR purification spin kit (Qiagen, Valencia, CA) according to the manufacturer’s protocol. DNA was suspended in sterile water and centrifuged repeatedly to remove residual resin before microinjection.

Generation and Identification of Transgenic Mice. Donor embryos for injection were generated by mating FVB/N male mice (National Cancer Institute Research Facility, Frederick, MD) to superovulated ICR female mice (Harlan Sprague Dawley, Indianapolis, IN) and were isolated as described (15). HK14.src530 or BK5.srcwt construct DNAs were injected into the pronuclei of these F1 embryos; survivors were transferred to either ICR (HK14.src530) or FVB/N (BK5.srcwt) pseudopregnant female mice. A construct containing a tyrosinase mini-gene (16) was co-injected with the HK14.src530 construct as described previously (17). HK14.src530 or BK5.srcwt construct DNAs were injected into the pronuclei of F1 embryos; survivors were transferred to either ICR (HK14.src530) or FVB/N (BK5.srcwt) pseudopregnant female mice. A construct containing a tyrosinase mini-gene (16) was co-injected with the HK14.src530 construct as described previously (17). Expression of the tyrosinase mini-gene confers a tan coat, as well as pigmentation of the eyes, ears, and tail to aid in the identification of transgenic animals. Transgenic animals were confirmed by PCR analysis of genomic DNA isolated from tail clips. The primers used to detect the BK5.srcwt transgene were rabbit β-globin intron specific oligos 5’GTGTTTGGATGAGAAGGT-3’ and 5’-TAAA-GAGAAAAGGATTGA-3’, which yielded a 390-bp fragment. Founder mice were mated back to the appropriate background strain to generate transgenic animals for additional experiments.

Analysis of Transgene Expression. Transgene expression was determined by indirect immunofluorescence analysis of histological sections from dorsal skin. The sections were fixed in formalin, embedded in paraffin, and 4 μm sections were adhered to slides. After deparaffinization, the slides were microwaved twice for 5 min each time to enhance staining. The sections were incubated with 10% nonimmunized goat serum for 20 min to block the nonspecific Fc receptor in the tissue, and then washed three times with PBS (pH 7.5), containing 1% BSA. The sections were then incubated with a 1:200 dilution of the primary rabbit anti-c-src polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in BSA/PBS for 1 h. After three washes with PBS containing 1% BSA, the sections were incubated with the secondary fluoroscent (FITC or Cy-3)-conjugated affinity pure F(ab)’s 2 fragment goat antirabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA; diluted 1:200) for 40 min. The sections were covered with VECTASHIELD mounting medium (Vector Laboratories, Burlingame, CA) before the coverslips were attached.

Histological Analyses. Dorsal skin samples and tumors were fixed in formalin and embedded in paraffin before sectioning. Sections of 4 μm were cut and stained with H&E. Mice received an i.p. injection of BrdUrd in PBS (100 μg/g body weight) 30 min before sacrifice. To analyze epidermal L1, paraffin sections were stained with an anti-BrdUrd antibody as described previously (16). For analysis of the expression of loricrin, and keratins 1, 5, and 6, the tissues were fixed in ethanol and immunostained as described previously (17).

For the analysis of response to TPA, adult female BK5.srcwt mice derived from lines A, B, and C were treated with a depilatory agent followed by washing. Note the spontaneous SCC on the back (arrowhead). E, Western blot analyses of c-src in the whole skin of adult BK5.srcwt transgenic (Tg) mice from lines A, B, and C, and age-matched nontransgenic (nTg) littersmates. Protein was normalized to β-actin.

Fig. 1. A, DNA construct used to generate HK14.src530 transgenic mice. The construct contains the HK 14 promoter, a generic intron, the human src530 mutant cDNA insert, and the human GH (growth hormone) polyadenylation signal. B, HK14.src530 transgenic mouse at 24–36 h after birth (top) and age-matched nontransgenic littermate (bottom). The transgenic mouse had thick and stunted ears. C and D, HK14.src530 transgenic mouse at 7 days of age (right) and age-matched nontransgenic littermate (left). The transgenic mouse had thick and stunted ears. E and F, H&E-stained sections of dorsal skin from 7-day-old nontransgenic and HK14.src530 transgenic mice, respectively. ×150. G and H, BrdUrd-stained sections of dorsal skin from 7-day-old nontransgenic and HK14.src530 transgenic mice, respectively; ×150.
from founder B and nontransgenic littermates were shaved on the dorsal side and treated topically 2 days later with either 1.7 or 3.4 nmol TPA or the acetone vehicle (0.2 ml). Treatments were given twice weekly over a 2-week period (four doses total), and mice were sacrificed at 48 h after the last treatment. Thirty min before sacrifice, mice received an i.p. injection of BrdUrd as described above. The dorsal skin was removed, fixed in formalin, embedded in paraffin, and then processed for conventional H&E staining and BrdUrd labeling. The determinations of epithelial thickness and LI were performed as described previously (18).

**In Vitro Src Kinase Assay.** Transgenic and nontransgenic mice were killed by cervical dislocation, and the dorsal skins were treated with a depilatory agent followed by washing. The skin was excised and frozen in liquid nitrogen. The frozen skin pieces were ground with a mortar and pestle, and lysed in lysis buffer [50 mM Tris-HCl (pH 7.4), 1% NP40, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 2 mM Na3VO4, and 1 mM NaF]. The whole skin lysates were homogenized with an 18-gauge needle/3-ml syringe before centrifugation at 14,000 × g for 15 min at 4°C. The supernatant was incubated with protein G-plus agarose (Oncogene Science, Cambridge, MA) for 10 min at 4°C followed by centrifugation at 14,000 × g for 10 min. src kinase activity was determined using a src assay kit (Upstate Biotechnology, Lake Placid, NY), which uses a synthetic peptide (KVEKIGEGTYGVVYK) as the substrate.

**RESULTS**

**Generation, Gross Physical Characteristics, and Histological Evaluation of HK14.src530 Transgenic Mice.** Persistent expression of src530 in the basal compartment of mouse epidermis was accomplished using the HK14.src530 construct described above. Six founders were obtained that carried the transgene, and all of the founders presented morphological differences that distinguished them from nontransgenic mice. The gross physical characteristics of founders were similar, as was the appearance of their skins at the microscopic level. Most of the founders died within 2–3 weeks of age. One founder that had a slightly milder phenotype survived and passed the transgene to offspring. However, all of the offspring from this founder possessed a more severe phenotype and died within 10 days after birth. This founder also died eventually from a SCC that developed spontaneously on the dorsal skin at the site of a small bite wound.

Fig. 1B shows the physical characteristics of HK14.src530 transgenic mice. Fig. 1B shows a HK14.src530 transgenic mouse 24–36 h after birth. The transgenic mouse had black eyes as a result of the cointegration (and cointegration) of the tyrosinase mini-gene along with the HK14.src530 transgene. These physical features made the initial identification of transgenic mice relatively easy. Fig. 1. C and D, show the characteristics of a HK14.src530 transgenic mouse at 7 days of age. The ears of these transgenic mice were very thick and stunted (Fig. 1C).

Histological evaluation of skin from HK14.src530 transgenic mice showed dramatic alterations in the epidermis. Fig. 1, E and F, show the H&E stained sections from a nontransgenic mouse at day 7 after birth and an age-matched transgenic mouse, respectively. Note the dramatic epidermal hyperplasia (involving both the interfollicular and follicular epidermis) and hyperkeratosis in the skin of these HK14.src530 transgenic mice. Fig. 1, G and H, shows sections stained for BrdUrd from these same mice. The LI was significantly higher in the interfollicular epidermis of this transgenic mouse (Fig. 1H) versus the nontransgenic littermate (Fig. 1G) because of the overexpression of the src530 mutant cDNA (42 ± 3.4 versus 3.6 ± 1.7; P < 0.05).

**Generation and Gross Physical Characteristics of BK5.srcwt Transgenic Mice.** To generate BK5.srcwt transgenic mice, a construct was made by inserting the wild-type human c-src cDNA into the BK5 expression vector as shown in Fig. 2A. This vector uses rabbit β-globin intron sequences as well as a SV40 polyadenylation signal to provide optimal expression of the inserted cDNA to the epidermal basal cells of the skin. Four founders with various degrees of phenotypic expression were obtained that carried the transgene as determined by PCR analysis of tail DNA, and three lines were established. Founder D died at 5 days of age because of the severity of the phenotype (Fig. 2B). This transgenic mouse was much smaller in size than normal, and the skin was very thick and scaly. Transgenic mice derived from founder C exhibited a scaly and thickened skin at 5 days of age, and older mice had an abnormal hair coat (Fig. 2C). These mice started to develop spontaneous SCCs at ~3 months of age (Fig. 2D). Lines derived from founders A and B did not have a marked skin phenotype, but human c-src protein levels were significantly elevated in epidermis of line B mice relative to nontransgenic littermates as assessed by Western blot analysis (Fig. 2E). The stronger phenotype in line C mice correlated with a higher level of human srcwt protein expression compared with line B mice (4.4-fold versus 11.5-fold, respectively). Lines B and C were used for additional experiments as described below.

**Histological Evaluation of Skin from the BK5.srcwt Founder D Transgenic Mouse.** Histological evaluation of skin from BK5.srcwt founder D showed dramatic alterations compared with nontransgenic littermates. Fig. 3, A and B, shows the H&E stained sections of skin from a nontransgenic mouse and founder D, respectively, at day 5 after birth. The epidermis of this BK5.srcwt founder showed severe hyperplasia, and the dermis and hypodermis were compressed. Immunostaining of skin sections and sections from other collected tissues showed high expression of the srcwt transgene in epidermis (Fig. 3D) and in the epithelial component of thymus, esophagus, trachea, uterine cervix, and vagina (data not shown). Keratin 6 was dramatically expressed in all layers of the interfollicular epidermis of founder D (Fig. 3F). Keratin 6 is normally only expressed in the outer root sheath of hair follicles in adult mice as shown in Fig. 3E. However, in response to wounding or treatment with proliferative stimuli such as TPA, K6 is expressed in interfollicular epidermis (20, 21). Therefore, K6 is thought to be a marker for proliferating interfollicular epidermal cells. Collectively, the above results indicated that overexpression of c-src led to epidermal hyperproliferation.

The expression pattern of several differentiation markers including loricrin, and keratins 1 and 5 are also shown in Fig. 3, H, J, and L,
respectively. In the skin of an age-matched nontransgenic littermate, loricrin was expressed in the granular layer (Fig. 3G), K1 was expressed in the spinous layer (Fig. 3I), and K5 was expressed in the basal layer of the interfollicular epidermis, and in the outer root sheath of hair follicles (Fig. 3K). In contrast, K1 expression was detected throughout the suprabasal compartment (Fig. 3J), and K5 expression was detected in all cell layers of the interfollicular epidermis (Fig. 3L) in founder D. Loricrin expression was limited to the granular layer (Fig. 3H) similar to the pattern observed in nontransgenic mice (Fig. 3G). These results also indicated that constitutive expression of human wild-type c-src led to epidermal hyperproliferation and a possible delay in epidermal differentiation.

**Histological Evaluation of Skin from BK5.src<sup>wt</sup> Transgenic Mice Derived from Founder C.** Histological evaluation of skin from BK5.src<sup>wt</sup> line C transgenic mice showed dramatic hyperplasia and hyperkeratosis (Fig. 4B). This phenotype was associated with the overexpression of the src<sup>wt</sup> transgene in the epidermis as detected by
Characterization of Spontaneous Skin Carcinogenesis in BK5.src<sup>wt</sup> Line C Transgenic Mice. Spontaneous SCCs started to develop in line C mice at ~3 months of age (Fig. 2D; Fig. 5A). The incidence of SCCs in a group of 30 line C BK5.src<sup>wt</sup> transgenic mice housed for ~2 years was 70% by 1 year (21 of 30 mice with one or more SCC). At the end of this experiment, 83.3% (25 of 30) of the transgenic mice had SCCs (Fig. 5A). In contrast, 0 of 30 nontransgenic littermates carried for the same period developed spontaneous skin tumors. Fig. 5B shows an H&E stained section of a SCC from a line C transgenic mouse. Fig. 5C shows the dramatic expression of human c-src in this tumor.

Fig. 5D shows src kinase activity in skin and SCCs from line C transgenic mice compared with the skin from nontransgenic littermates. Src kinase activity was significantly higher in skin and SCCs of line C mice compared with the activity in skin of nontransgenic mice (P = 0.0495; Mann-Whitney U test).

Analysis of TPA Responsiveness in BK5.src<sup>wt</sup> Transgenic Mice Derived from Founder B. Hemizygous and homozygous BK5.src<sup>wt</sup> line B transgenic mice did not show a marked skin phenotype as noted above. In this regard, there were no significant differences in epidermal thickness (see Fig. 6, A and B; Fig. 7A) and LI (see Fig. 6, C and D; Fig. 7B) between transgenic mice and nontransgenic littersmates as assessed in H&E- and BrdUrd-stained skin sections, respectively. However, as noted above, line B mice had elevated c-src protein in the skin as determined by Western blot analysis (see again Fig. 2E).

As shown in Figs. 6 and 7, BK5.src<sup>wt</sup> line B transgenic mice were analyzed for their responsiveness to the phorbol ester skin tumor promotor, TPA. Epidermal hyperplasia (measured as epidermal thickness) and epidermal LI were assessed in line B transgenic mice and nontransgenic littersmates after topical treatment with 1.7 or 3.4 nmol TPA, or 0.2 ml of the acetone vehicle. For this experiment, adult hemizygous transgenic mice and nontransgenic littersmates received four applications of TPA or vehicle, given twice weekly, and were sacrificed at 48 h after the last treatment. Fig. 6, E and F, show the H&E stained sections from a nontransgenic and a line B transgenic mouse, respectively, treated with 3.4 nmol TPA. Fig. 6, G and H, show the corresponding Brdu-stained sections. As summarized in Fig. 7, A and B, both the epidermal thickness and LI were significantly higher in transgenic mice treated with TPA than in the nontransgenic littersmates (Fig. 6, E and G), and with the exception of LI at a dose of 3.4 nmol TPA, these differences were statistically significant (P < 0.05; Mann-Whitney U test). TPA treatment led to elevated src kinase activity in whole skin lysates from both nontransgenic and line B transgenic mice. The higher src kinase activity in lysates from line B mice treated with TPA was statistically significant (P < 0.05; Mann-Whitney U test) as shown in Fig. 7D. These results indicate that elevated c-src activity in the epidermis of line B mice was associated with a hypersensitivity to the effects to TPA.

Responsiveness of BK5.src<sup>wt</sup> Line B Transgenic Mice to Two-Stage Carcinogenesis. To determine the responsiveness of BK5.src<sup>wt</sup> line B transgenic mice to two-stage carcinogenesis, three groups (28 mice each) of transgenic and nontransgenic mice were treated as follows: (a) acetone at initiation followed 2 weeks later by twice-weekly applications of 6.8 nmol TPA; (b) DMBA initiation (100 nmol) followed 2 weeks later by twice-weekly applications of 3.4 nmol TPA; and (c) DMBA initiation (100 nmol) followed 2 weeks later by twice-weekly applications of 6.8 nmol TPA. TPA treatment was continued for 80 weeks, during which time the incidence and multiplicity of papillomas and SCCs were scored in each group. The results from this experiment are shown in Fig. 8. Note that no spontaneous skin tumors were detected in untreated line B transgenic mice. The incidence of papillomas reached a plateau by ~15 weeks in both transgenic and nontransgenic mice that had been initiated with...
DPBA and promoted with 6.8 nmol TPA (Fig. 8A). In the groups of mice initiated with DMBA and promoted with 3.4 nmol TPA, papillomas were visible on the skin of BK5.srcwt line B transgenic mice by 5 weeks of promotion, and by 12 weeks of TPA treatment, 100% of the transgenic mice exhibited tumors (average of 6.4 papillomas/mouse); however 96% (4.6 papillomas/mouse) of the nontransgenic mice also developed papillomas. This result suggested that promotion with 6.8 nmol TPA might have been too high to

TPA were significant at 5–23 weeks during the experiment as shown in Fig. 8A (P < 0.04; χ² test), and the differences in papilloma multiplicity between nontransgenic and transgenic mice were significant at 5–23 weeks during the experiment as shown in Fig. 8B (P < 0.001; Mann-Whitney U test).

In the groups of mice initiated with DMBA and promoted with 6.8 nmol TPA, papillomas were visible on the skin of BK5.srcwt line B transgenic mice by 5 weeks of promotion, and by 12 weeks of TPA treatment, 100% of the transgenic mice exhibited tumors (average of 6.4 papillomas/mouse); however 96% (4.6 papillomas/mouse) of the nontransgenic mice also developed papillomas. This result suggested that promotion with 6.8 nmol TPA might have been too high to
Fig. 8. Effect of transgene expression on induced tumorigenesis in skin using BK5.src wt line B transgenic mice and corresponding nontransgenic littermates. A, percentage of mice with papillomas (tumor incidence) within 23 weeks of promotion with 3.4 nmol or 6.8 nmol TPA given twice weekly. B, average number of papillomas per mouse (tumor multiplicity) within 23 weeks of promotion with 3.4 nmol or 6.8 nmol TPA given twice weekly. C, percentage of mice with SCCs (incidence) within 80 weeks of promotion with 3.4 nmol or 6.8 nmol TPA given twice weekly. D, average number of SCCs per mouse (multiplicity) within 80 weeks of promotion with 3.4 nmol or 6.8 nmol TPA given twice weekly. Treatments were as follows: (●), BK5.src wt line B transgenic mice initiated with 100 nmol DMBA and promoted with 6.8 nmol of TPA; (○), nontransgenic littermates initiated with 100 nmol DMBA and promoted with 6.8 nmol of TPA; (△), transgenic mice initiated with 100 nmol DMBA and promoted with 3.4 nmol of TPA; (▽), nontransgenic littermates initiated with 100 nmol DMBA and promoted with 3.4 nmol of TPA; (◆), transgenic mice that received the acetone vehicle (0.2 ml) at initiation followed by promotion with 6.8 nmol of TPA; (□), nontransgenic littermates that received the acetone vehicle (0.2 ml) at initiation followed by promotion with 6.8 nmol of TPA; (▲), nontransgenic mice initiated with 100 nmol DMBA and promoted with 6.8 nmol TPA; (■), nontransgenic littermates initiated with 100 nmol DMBA and promoted with 6.8 nmol TPA; (□), transgenic mice that received the acetone vehicle (0.2 ml) at initiation followed by promotion with 6.8 nmol of TPA. E, Protein expression and src kinase activity in papillomas (paps) and SCCs induced by initiation with DMBA followed by promotion with TPA in nontransgenic (NTg, □) and BK5.src wt transgenic (Tg, ■) mice. Total kinase activity relative to the activity in NTg papillomas is shown. Whole cell lysates were immunoprecipitated using agarose conjugated monoclonal antibodies to v-src. The immunoprecipitates were subjected to Western blot analysis and assayed for src kinase activity.

evaluate the difference in responsiveness between BK5.src wt transgenic mice and nontransgenic littermates to two-stage carcinogenesis. However, in these groups, papillomas arose earlier in transgenic mice than in nontransgenic mice. The differences in papilloma incidence between nontransgenic and transgenic mice were significant at selected time points (5 and 6 weeks) during the experiments as shown in Fig. 8A (P < 0.03; χ² test), and the differences in papilloma multiplicity between nontransgenic and transgenic mice were also significant at selected early time points (5, 6, 8–11, and 13 weeks) during the experiment as shown in Fig. 8B (P < 0.05; Mann-Whitney U test). These data with BK5.src wt line B transgenic mice are consistent with recent results obtained with HK1.src529 transgenic mice (13), and demonstrate that elevated c-src kinase activity in epidermis leads to enhancement of TPA-mediated skin tumor promotion.

Notably, SCCs developed very rapidly and in greater number in BK5.src wt line B transgenic mice as shown in Fig. 8, C and D. In the groups of transgenic mice initiated with DMBA and promoted with either 3.4 or 6.8 nmol TPA, the incidence of SCCs reached 100% by 37 weeks of promotion in both groups. In addition, an average of ~4 SCCs/mouse developed in both groups of transgenic mice. In contrast, nontransgenic mice developed significantly fewer SCCs per mouse (1.0 and 1.5, for the 3.4 and 6.8 nmol groups, respectively). As shown in Fig. 8E, total src kinase activity was significantly elevated in papillomas and SCCs from transgenic mice initiated with DMBA and promoted with 3.4 nmol TPA compared with papillomas and SCCs from nontransgenic mice. Additional analyses of transgenic mice that developed SCCs revealed that metastases to peripheral lymph nodes (Fig. 9, A and B) were significantly higher than in nontransgenic mice as summarized in Table 1 (P = 0.0085; Mann-Whitney U test). In addition, metastases to other organs such as lung (Fig. 9C), pleura (Fig. 9D), and to mediastinal lymph nodes were observed only in transgenic mice (Table 1). Thus, higher levels of src protein and kinase activity in tumors of line B transgenic mice correlated with a more rapid progression rate and a higher rate of metastasis compared with tumors generated in nontransgenic littermates.

DISCUSSION

In the current study, we generated several new lines of transgenic mice that overexpress wild-type human c-src using the BK5 promoter that targets expression to the basal layer of the interfollicular epidermis, the outer root sheath of hair follicles, and the epithelial cells that line the sebaceous glands (22, 23). In addition, use of the BK5 promoter leads to constitutive expression in adult mice (23). Recently, we generated HK1.src529 transgenic mice that overexpress a constitutively active mouse c-src mutant (src529) in interfollicular epidermis driven by the HK1 promoter (13). These mice developed significantly greater epidermal hyperplasia and hyperkeratosis than nontransgenic
littermates for ~1 week after birth; however, this phenotype did not persist into adulthood. These mice also displayed enhanced sensitivity to skin tumor promotion by TPA, but did not develop any spontaneous skin tumors, and we did not examine the impact of elevated c-src activity on tumor progression in these mice. The current results demonstrate for the first time that elevated c-src activity in skin epidermis of transgenic mice leads to spontaneous development of SCCs. In addition, the current results demonstrate for the first time that elevated c-src activity in skin papillomas enhances their progression to malignancy (SCCs) and additionally enhances the ability of SCCs to metastasize to lymph nodes and distant organs. Finally, the current results confirm that elevated c-src activity in epidermis enhances responsiveness to the skin tumor promoter TPA suggesting a role for this nonreceptor tyrosine kinase in several stages of multistage skin carcinogenesis.

Many lines of evidence have suggested that c-src is involved in the genesis and progression of multiple types of human cancer (24). Analysis of cultured tumor cell lines and surgically generated tumor tissue has shown that protein expression and/or kinase activity of c-src is elevated in neuroblastomas, myeloproliferative disorders, and carcinomas of the colon, breast, lung, esophagus, skin, uterine cervix, and gastric tissues (24). In addition, continued increases in c-src expression and activity are associated with disease progression (24, 25). The development and characterization of relevant animal models have been suggested as a promising way to test the hypotheses derived from analysis of human tumor tissues (24). In the current study, BK5.src<sup>wt</sup> line B transgenic mice exposed to an initiation/promotion protocol using DMBA/TPA developed tumors considerably faster and in greater numbers than similarly treated nontransgenic mice, especially at the lower dose of TPA (3.4 nmol). This dramatic increase in tumor formation in BK5.src<sup>wt</sup> line B transgenic mice may be explained, in part, by their enhanced sensitivity to the tumor promoter, TPA. As shown in Fig. 7, A and B, BK5.src<sup>wt</sup> line B transgenic mice showed an exaggerated hyperplasia and cell proliferation (as measured by LI) after TPA treatment compared with nontransgenic mice. An increase in epidermal hyperplasia and in LI are hallmarks of tumor promotion in mouse skin (26). The increased sensitivity to TPA promotion may be attributable primarily to the fact that TPA up-regulates src kinase activity in nontransgenic mice (Ref. 13; see Fig. 7D) and, thus, was additionally up-regulated in transgenic mice during TPA treatment.

The exact mechanism(s) for the enhanced sensitivity of BK5.src<sup>wt</sup> line B transgenic mice to the proliferative effects of TPA is not clear. One possible mechanism may be because of enhanced cooperativity between c-src and EGFr signaling pathways. Work in our laboratory has shown that topical application of TPA and other tumor promoters elevated expression of EGFr ligands including TGF-α, amphiregulin, and heparin-binding EGFr mRNA, and protein in mouse epidermis (27, 28). Furthermore, elevated heterodimer formation between the EGFr and erbB2 was observed in epidermis treated with TPA (12). Evidence in the literature has suggested that c-src can associate with erbB2 (29, 30) and EGFr (31, 32). It is possible that c-src activation in mouse epidermis can cooperate with EGFr signaling pathways, which ultimately cooperate in altering expression or function of genes responsible for cell proliferation, a requirement for tumor promotion. One such pathway may involve activation of STATs. Very recently we have found that members of the STAT family become activated in TPA-treated epidermis, including STATs 1, 3, and 5 (33). One mechanism for activation of STATs involves the interaction among EGFr, c-src, and STATs (34, 35). In addition, Bao et al. (36) reported recently that src promotes the destruction of c-cbl, a regulator of EGFr endocytosis, thereby enabling EGFr to escape desensitization prolonging its signaling. Future studies will explore whether STATs are activated in epidermis of BK5.src<sup>wt</sup> line B transgenic mice after TPA treatment and the role of STATs in enhanced sensitivity of these mice to tumor promotion.

A very interesting finding in the current study was that elevated src kinase activity in papillomas led to very rapid progression of these tumors to SCCs. In addition, elevated src kinase activity in SCCs led to enhanced metastasis to lymph nodes and distant organs. In two-stage carcinogenesis experiments, papilloma progression or malignant conversion is highly dependent on the tumor burden (37) and on the genetic background of the strain used to induce tumors (38–40). All three lines of BK5.src<sup>wt</sup> transgenic mice were generated and maintained on an FVB/N genetic background, which has been shown by Hennings et al. (39) to be relatively genetically prone to malignant conversion compared with other inbred and outbred lines of mice used for two-stage carcinogenesis experiments (e.g., SENCAR). However, the presence of elevated src kinase activity in skin papillomas generated on an FVB/N genetic background led to a significant enhancement of malignant conversion and metastasis. c-src has been implicated in the progression and metastasis of many human cancers (reviewed in Ref. 25).

Whereas the exact mechanism(s) for these effects of src are not well understood, src has been shown to activate and/or modulate a number of targets that can lead to enhanced progression and metastasis, including activation of the Ras/mitogen-activated protein kinase pathway (41), activation of STAT3 (42), association with adhesion molecules such as catenins and cadherins (43, 44), up-regulation of vascular endothelial growth factor (45, 46), and up-regulation of...

<table>
<thead>
<tr>
<th>Mice</th>
<th>Incidence of peripheral LN metastasis&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Number of metastatic LNs</th>
<th>Other organs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nontransgenic</td>
<td>11/22 (50%)</td>
<td>0.73 ± 0.21</td>
<td>Lung (3)</td>
</tr>
<tr>
<td>Transgenic</td>
<td>15/20 (75%)</td>
<td>1.85 ± 0.33</td>
<td>Pleura (1)</td>
</tr>
</tbody>
</table>

<sup>a</sup> LN, lymph node.
<sup>b</sup> Number of mice with axillary and/or inguinal LN metastases/number of mice with SCCs.

<sup>c</sup> P = 0.0085 (Mann-Whitney U test).
Bcl-X\(_L\), a STAT3 regulated antiapoptotic protein (47). As noted above, we have found that STAT3 is activated in TPA-treated mouse epidermis, and also shows up-regulated in skin papillomas and SCCs induced by DMBA/TPA two-stage carcinogenesis protocols (33). Thus, elevated src kinase activity in papillomas and SCCs from BK5.src\(^{wt}\) line B transgenic mice appear to provide the up-regulation/ modulation of several target molecules that lead to enhancement of malignant conversion and metastasis in this model of epithelial carcinogenesis. We are currently examining these and other possibilities.

In the current study, we also found that spontaneous SCCs developed in BK5.src\(^{wt}\) line C transgenic mice in the absence of any carcinogen or tumor promoter treatment. Of the three lines established from the BK5.src\(^{wt}\) construct, line C mice had the strongest phenotype. A significant proportion of the SCCCs appeared to arise at sites of wounding. In addition, we reported recently that SCCCs developed at the site of tissue biopsies following the induction of c-src expression in c-src gene-switch transgenic mice (48). These data support the hypothesis that c-src can function as an oncogene under certain circumstances where a second stimulus such as wound healing and/or tumor promoter treatment is present. With regard to the latter point, analysis of the data in Fig. 8 shows that ~75% of line B mice treated only with TPA developed SCCCs with an average of 1.7 SCCs/mouse. Thus, elevated c-src protein and kinase activity appeared to substitute for an initiating event in epidermis of FVB/N mice. Additional work exploring the oncogenic functions of c-src in this model system would seem warranted.

To date, two other groups have reported transgenic mouse overexpressing src. In one group, MMTV.c-src transgenic mice (49) and GFAP.c-src (50). In MMTV.c-src transgenic mice that carried a constitutively activated form of c-src under transcriptional control of the MMTV long-terminal repeat, the induction of focal mammary epithelial hyperplasias was detected as early as 2 months of age. These lesions progressively became more involved as the animals aged, and focal mammary tumors began to appear as early as 7 months of age. By 10–12 months of age, 50% of the female transgenic mice had developed tumors (49). In GFAP.c-src transgenic mice that overexpressed the v-src kinase under control of the GFAP gene regulatory elements in astocytes, abnormal gliosis was observed in all of the transgenic animals at 2 weeks of age, frequently followed by the development of dysplastic changes, and overt astrocytoma developed in the brain and spinal cord (~15% at 16 months of age; Ref. 50). These studies indicate that elevated and/or deregulated src kinase activity in several tissues can lead to spontaneous tumor development. Data obtained in BK5.src\(^{wt}\) line C transgenic mice are consistent with these earlier reports. An apparent advantage of the current work was that overexpression of wild-type c-src led to development of a transgenic line (line B) that could be used for chemical carcinogenesis experiments.

In conclusion, we have developed a new mouse model, BK5.src\(^{wt}\) transgenic mice, to study the role of c-src in multistage epithelial carcinogenesis. In these mice, overexpression of human c-src led to enhanced tumor promotion by TPA, a finding consistent with our recent data using HK1.src\(^{529}\) transgenic mice (13). In addition, elevated levels of src protein and kinase activity led to rapid conversion of papillomas to SCCs that exhibited an enhanced metastatic phenotype compared with tumors generated in nontransgenic mice. BK5.src\(^{wt}\) transgenic mice appear to be a unique animal model to additionally study the mechanisms whereby elevated c-src kinase activity contributes to the process of multistep carcinogenesis.

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REFERENCES


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